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# Induction of Neutralizing Antibodies and Th1-Polarized and CD4-Independent CD8$^+$ T-Cell Responses following Delivery of Human Immunodeficiency Virus Type 1 Tat Protein by Recombinant Adenylate Cyclase of *Bordetella pertussis*

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**HIV-Tat, a conserved protein playing a key role in the early life cycle of the human immunodeficiency virus (HIV) has been proposed as a potential AIDS vaccine. An HIV-Tat-based vaccine should elicit a broad, long-lasting, and neutralizing immune response. We have previously demonstrated that the adenylate cyclase (CyaA) from *Bordetella pertussis* targets dendritic cells and delivers CD8$^+$ and CD4$^+$ T-cell epitopes into the major histocompatibility complex class I and class II presentation pathways. We have also showed that CyaA induced specific and protective cytotoxic T cell responses in vivo. Here, we designed a prototype vaccine based on the HIV type 1 Tat delivered by CyaA (CyaA-E5-Tat) and tested its capacity to induce HIV-Tat-specific cellular as well as antibody responses. We showed that immunization of mice by CyaA-E5-Tat in the absence of adjuvant elicited strong and long-lasting neutralizing anti-Tat antibody responses more efficient than those obtained after immunization with Tat toxoid in aluminum hydroxide adjuvant. Analyses of the anti-Tat immunoglobulin G isotypes and the cytokine pattern showed that CyaA-E5-Tat induced a Th1-polarized immune response in contrast to the Th2-polarized immune responses obtained with the Tat toxoid. In addition, our data demonstrated that HIV-Tat-specific gamma interferon-producing CD8$^+$ T cells were generated after vaccination with CyaA-E5-Tat in a CD4$^+$ T-cell-independent manner. Based on these findings, CyaA-E5-Tat represents an attractive vaccine candidate for both preventive and therapeutic vaccination involving CyaA as an efficient nonreplicative vector for protein delivery.**

Since the discovery of human immunodeficiency virus type 1 (HIV-1) as the causative agent of AIDS, attempts to develop therapeutic or prophylactic vaccination to contain the virus have failed. Recently, different reports have suggested that an HIV-Tat (Tat)-based vaccine might be an attractive option (23, 24, 33, 35). Indeed, the Tat protein is critical in the development of the AIDS disease (3, 29). This regulatory protein is expressed very early in the viral life cycle, even prior to virus integration (71). Following extracellular secretion by infected cells, Tat is taken up by neighboring cells (21, 22, 31) that, in turn, up-regulate expression of chemokine receptors, increasing their susceptibility to the viral infection (41, 63). Tat also enhances dendritic cell (DC) maturation (25) and induces expression of chemokines that contribute to the recruitment of activated T cells and macrophages, the targets of HIV-1 infection (44).

Tat is relatively well conserved among various HIV strains, and a correlation between the rise of anti-Tat antibodies and nonprogression to AIDS has been established (55, 56, 73). In addition, a recent study has pointed out the relevance of anti-Tat cytotoxic T lymphocyte (CTL) responses to control the early virus infection (1). Recent data have demonstrated that a Tat DNA-based vaccine or a mucosal administration of active Tat protein using adjuvant induce both humoral and cellular anti-Tat immune responses in mice (7, 10, 11, 49). In preclinical studies with macaques, immunization with Tat in the presence of adjuvant also protected animals from simian human immunodeficiency virus 89.6 infection (9). However, based on the possible toxic and immunosuppressive effects of native Tat (14, 53, 69, 70, 72), its utilization as a vaccine antigen raises serious safety concerns. An inactive form was recently prepared by alkylation of cysteines residues, and immunization of macaques with this Tat toxoid concomitantly with adjuvant only attenuated virus replication (52, 67). The safety and immunogenicity of Tat toxoid was also demonstrated in immunocompromised HIV-1-infected individuals (36).

Thus, a Tat-based vaccine should elicit a broad, long-lasting, and neutralizing immune response. Toward this goal, we have developed a new strategy of vaccination that targets professional antigen-presenting cells (APCs) (28). The adenylate cyclase (CyaA), a 1,706-amino-acid-residue protein produced by *Bordetella pertussis*, binds specifically to the β2 integrin CD11b expressed on APCs such as DCs (38). We have designed a nonreplicative vector based on a genetically detoxified CyaA. Its ability to deliver CD8$^+$ and CD4$^+$ epitopes, respectively, into the major histocompatibility complex (MHC) class I and class II presentation pathways has been extensively docu-

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mented (16, 27, 28, 39, 45, 47, 60). Preclinical experiments with mice have also demonstrated that the CyaA vector is able to prime specific protective and therapeutic T-cell immunity against virus or against tumor cell development (26, 59). So far, however, these studies have been limited to the analysis of T-cell responses. Thus, the capacity of CyaA to induce or potentiate specific B-cell responses through antigen delivery to DC remains to be assessed.

Here, we report the design and characterization of a CyaA recombinant molecule carrying the full-length sequence of the HIV-1 Tat protein (CyaA-E5-Tat) inserted between residues 224 and 225 of its catalytic domain. First, we showed that the insertion of Tat into CyaA does not affect its targeting to CD11b-expressing cells but inhibits the transactivating activity of Tat. Immunization with CyaA-E5-Tat elicited an increased Tat-specific neutralizing antibody production compared to that observed with Tat toxoid in alum. Remarkably, humoral and cellular immune responses were long lasting. Analyses of the anti-Tat immunoglobulin G (IgG) isotypes and of cytokine production demonstrated that CyaA-E5-Tat triggers the development of primary responses toward the Th1 type as opposed to the Th2 responses observed with Tat toxoid in alum. In addition, we showed that vaccination with CyaA-E5-Tat primes Tat-specific gamma interferon (IFN-γ)-producing $CD8^+$ T cells independently of $CD4^+$ T cell help. Noticeably, this is the first report demonstrating that the CyaA vector is able to deliver a full protein to induce higher antibody responses than those observed after immunization with the protein alone. These findings will contribute to the definition of new strategies in vaccination involving CyaA as a safe and efficient nonreplicative vector for protein delivery inducing preventive or therapeutic antiviral vaccines.

## MATERIALS AND METHODS

**Mice and reagents.** Six- to eight-week-old BALB/c female mice were obtained from Janvier (Le Genest St. Isle, France). They were immunized intraperitoneally (i.p.) on days 0, 20, and 40 with 10 μg of CyaA-E5 or CyaA-E5-Tat in phosphate-buffered saline (PBS). Alternatively, mice were immunized subcutaneously (s.c.) on days 0, 20, and 40 with 10 μg of CyaA-E5 or CyaA-E5-Tat or with recombinant Tat toxoid (1 or 10 μg) in PBS or mixed with 120 μg of alum (Serva, Heidelberg, Germany). For intradermic immunization, mice were injected in the ear dermis with 10 μg of CyaA-E5 or CyaA-E5-Tat or Tat toxoid in PBS. For enzyme-linked immunospot assay, mice were immunized i.p. at days 0 and 20 with CyaA-E5 or CyaA-E5-Tat in alum (1 mg) as previously described (26, 28, 59) or with 10 μg of Tat toxoid in alum (1 mg). In vivo depletion of $CD4^+$ or $CD8^+$ T cells was carried out by i.p. injections of purified rat anti-CD4, anti-CD8, or control isotype monoclonal antibodies (MAbs; 300 μg/injection) (59) on days −1, 0, +1, 19, 20, and 21 or on days 28, 29, and 30. The depletion efficiency was checked by cytofluorimetric analysis. Depletion of $CD4^+$ or $CD8^+$ T cells was >95% after treatment with the appropriate MAb. Peptides (amino acids 26 to 34, 31 to 39, 36 to 44, 41 to 49, 11 to 24, 21 to 40, and 46 to 65) of the Tat protein (Neosystem, Strasbourg, France) were dissolved in PBS and kept at −20°C.

**Expression and purification of recombinant adenylate cyclase toxins and toxoids.** DNA manipulations were performed according to standard procedures (58) using the *Escherichia coli* XL1-Blue strain (Stratagene, Amsterdam, The Netherlands) as host cells. The plasmids coding for the wild-type CyaA (pT7CACT1) or the detoxified variant (by disrupting the adenylate cyclase enzymatic activity by insertion of the dipeptide Leu-Gln between codons 188 and 189 of CyaA) CyaA-E5 (pCACT-E5) have been previously described (57, 62). The plasmid pCACT-E5-Tat-OVA is a derivative of pCACT-E5-OVA (26) in which the sequence coding for the HIV-1 Tat protein (DNA from pTRCAG-Tat) (34) has been inserted between the BsiWI and NheI restriction sites. In the resulting protein, CyaA-E5-Tat, the Tat sequence is inserted between residues 224 and 225 of CyaA, in fusion with the OVA $CD8^+$ T-cell (SIINFEKL) epitope. The protocol for CyaA production has already been described elsewhere (45). All proteins were expressed in the *E. coli* BLR strain (Novagen, Merck KG. Darmstadt, Germany) and were purified to more than 95% homogeneity (as judged by sodium dodecyl sulfate [SDS] gel analysis) from inclusion bodies by a two-step procedure, including DEAE-Sepharose and phenyl-Sepharose chromatographies, as described before (37). An additional purification step was added to remove endotoxin contaminations (lipopolysaccharide). The purified protein was diluted in 20 mM HEPES, pH 7.5, and loaded again onto a clean phenyl-Sepharose column. The column was washed with 5 column volumes of 20 mM HEPES, pH 7.5, and then washed three times with 10 column volumes of 60% isopropanol in 20 mM HEPES, pH 7.5, and 10 column volumes of 20 mM HEPES, pH 7.5, alternatively. The recombinant CyaA protein was not eluted from the phenyl-Sepharose resin in the presence of 60% isopropanol, whereas endotoxins were washed away in these conditions (32). Finally, the protein was eluted in 20 mM HEPES, pH 7.5, 8 M urea. The final endotoxin concentration was determined by quantitative chromogenic LAL assay (QCL-1000; Bio-Whittaker) and found to be less than 1 endotoxin unit (EU)/μg of protein. Protein concentrations were determined spectrophotometrically from the absorption at 278 nm using a molecular extinction coefficient of 141 $mM^{-1}$ $cm^{-1}$. The HIV-1 Tat protein and Tat toxoid were produced and purified by Aventis Pasteur (Marcy l'étoile, France). The wild-type Tat protein (amino acid sequence identical to the Tat polypeptide inserted into CyaA-E5-Tat) was overproduced in *E. coli* and purified by ammonium sulfate precipitation and ion-exchange chromatography on SP-Sepharose fast flow. The chemically inactivated Tat toxoid was obtained by selective alkylation of the cysteine residues of the purified Tat. The endotoxin concentrations of the wild-type Tat and Tat toxoid were under 0.5 EU/μg.

**Immunoblotting.** After SDS-polyacrylamide gel electrophoresis, the recombinant CyaA proteins were electrotransferred to a nitrocellulose membrane (0.45 μm; Bio-Rad, Marnes la Coquette, France) which was probed with a mouse monoclonal anti-HIV-1 Tat antibody (AbCys SA, France). The immunoblot was detected with goat anti-mouse immunoglobulins conjugated to phosphatase alkaline (Chemicon, Temecula, CA) and revealed with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (Sigma, St. Louis, MO).

**Binding assays.** Chinese hamster ovary cells transfected with human CD11b/CD18 (CHO-CD11b cells) were cultured in the presence of Geneticin (1 mg/ml). The binding assays were performed as described previously (38). Briefly, $4 \times 10^5$ cells were incubated with the indicated concentrations of CyaA molecules in Dulbecco's modified Eagle's medium containing 4.5 mg/ml glucose (Invitrogen), without serum, in 96-well culture plates for 30 min on ice. Then, CyaA-biotin (30 nM) was added in the continuous presence of the recombinant CyaAs. After washing and removal of supernatants, the cells were stained with streptavidin-phycoerythrin (PharMingen). After the last wash, the cells were analyzed on a FACScan flow cytometer (Becton Dickinson) in the presence of 50 μg/ml propidium iodide to exclude dead cells. The binding data were deduced from the mean fluorescence intensity and analyzed as previously described (38).

**Analysis of antibody responses.** The mice were bled at days 20, 28, and 48 after immunization, and individual mouse sera were tested for antibody responses by enzyme-linked immunosorbent assay (ELISA). Microplates (Nunc) were incubated overnight with Tat toxoid (1 μg/ml) in PBS at 4°C. For epitope mapping, peptides (amino acids 1 to 20, 21 to 40, 36 to 50, 46 to 65, 56 to 70, 65 to 80, and 73 to 86) overlapping the HIV-1 Tat sequence (Neosystem, Strasbourg, France) were dissolved in PBS and kept at −20°C. All peptides (10 μg/ml) were incubated overnight in microplates in 50 mM sodium carbonate buffer at 4°C. After washes in PBS-Tween 20 (0.1%), diluted sera were added to the wells and incubated for 1 h at 37°C. Following washes in PBS-Tween 20 (0.1%), plates were incubated with goat anti-mouse IgG, IgG1, or IgG2a peroxidase conjugate (Sigma) for 1 h at 37°C. After washes in PBS-Tween 20 (0.1%), plates were developed using *o*-phenylenediamine and hydrogen peroxide (Sigma). The reaction was stopped with sulfuric acid, and the plates were analyzed at 492 nm in an ELISA reader (Dynatech, Marnes la coquette, France). The negative control consisted of sera from mice immunized with CyaA-E5. Results are expressed as antibody titers calculated by linear regression analysis plotting dilution versus $A_{492}$. The titers were calculated to be the $\log_{10}$ highest dilution that gave twice the absorbance of pooled control sera diluted 1/100. Titers are given for individual serum samples.

**Single IFN-γ-producing cell enzyme-linked immunospot assay.** Multiscreen filtration plates (96 wells; Millipore, Molshein, France) were coated overnight at room temperature with 4 μg of rat anti-mouse IFN-γ antibody (clone R4-6A2; PharMingen, San Diego, CA) per ml, and then the plates were washed and blocked with complete medium (RPMI 1640 medium with Glutamax I [Life Technologies, Inc., Grand Island, NY] supplemented with 10% fetal calf serum, antibiotics, and $5 \times 10^{-5}$ M β-2-mercaptoethanol). Serial twofold dilutions of

the spleen cell suspensions were added to the wells along with $5 \times 10^5$ γ-irradiated (2,500 rads) syngeneic spleen cells. The cells were incubated for 36 h at 37°C, 7.5% $CO_2$ with or without a pool of overlapping nonamer peptides of HIV-Tat (amino acids 26 to 34, 31 to 39, 36 to 44, and 41 to 49) (10 μg/ml for each peptide) or a pool of two longer peptides of HIV-Tat (amino acids 11 to 24 and 21 to 40) (10 μg/ml for each peptide) (49). After extensive washes, the plates were revealed by incubation with 4 μg/ml of biotinylated rat anti-mouse IFN-γ antibody (clone XMG 1.2; PharMingen) followed by incubation with streptavidin-alkaline phosphatase (PharMingen). Finally, spots were revealed using 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium as the substrate. The number of IFN-γ-producing cells was determined by counting the number of spot-forming cells (SFC) in each well (Bioreader, Karben, Germany), and the results are expressed as numbers of SFC per $10^6$ cells.

**Cytokine production assay.** At day 28 or 48, the spleens were removed and single-cell suspensions were cultured in complete medium. Cells were placed in a humidified atmosphere of 7.5% $CO_2$ in air. Different concentrations of the peptide (amino acids 46 to 65) of Tat (1 to 10 μg/ml) were added at the beginning of the culture (40). After 72 h, IFN-γ and interleukin-5 (IL-5) productions were determined in culture supernatants by ELISA as previously described (17). All assays were standardized with the corresponding recombinant murine cytokines (PharMingen).

**Neutralization assay.** The assay for the anti-Tat neutralizing capacity of the murine sera is based on the transactivating capacity of the native Tat to activate the production of β-galactosidase (β-Gal) in P4 cells, a kind gift of Olivier Schwartz (48). P4 cells are CD4-positive, HeLa cell-derived cells in which transactivation by Tat induces expression of the *Escherichia coli lacZ* gene from the HIV long terminal repeat (LTR). P4 cells were seeded ($5 \times 10^5$/ml) in 24-well plates in 1 ml of Dulbecco's modified essential medium (DMEM) with Glutamax I DMEM containing 10% fetal calf serum, antibiotics, and $5 \times 10^{-5}$ M β-2-mercaptoethanol. Recombinant native Tat kept at −80°C under deoxygenated atmosphere was diluted at 80 ng/ml in complete HL-1 medium (2 mM HL-1 medium containing glutamine [Life Technology], 0.1 mM chloroquine [Sigma], 0.05 mM β-mercaptoethanol [Life Technology], 1/200 Complete inhibitor [Roche Diagnostic], and 0.5% bovine serum albumin [Sigma]). The murine sera were pooled, diluted in complete HL-1 medium, and incubated with the native Tat for 20 min at 4°C. The mixture was then added to P4 cells for 2 h at 37°C, 7.5% $CO_2$. The medium was then replaced with DMEM containing antibiotics and $5 \times 10^{-5}$ M β-2-mercaptoethanol, and after overnight incubation, cells were harvested using 200 μl of PBS–5 mM EDTA for 5 min, washed in PBS, spun down at 13,000 rpm for 10 min, and analyzed by quantitative measurement of β-Gal expression at 595 mn using an ELISA reader. The value obtained with P4 cells incubated with recombinant Tat is considered 0% neutralization, whereas the value obtained with P4 cells cultured in medium is considered 100% neutralization.

**BM-DC generation and analysis of apoptosis.** The femurs and tibiae of 6- to 8-week-old BALB/c mice were removed and cleared from surrounding muscles. The bones were cut and placed in PBS, and the bone marrow was flushed out with a syringe. The bone marrow cells were spun down for 10 min at 13,000 rpm, counted, and plated in 10-cm-diameter petri dishes (Falcon) at a density of $2 \times 10^5$ cells per ml in RPMI 1640 with Glutamax I, supplemented with 10% fetal calf serum, antibiotics, $5 \times 10^{-5}$ M β-2-mercaptoethanol, and 4 ng/ml granulocyte-macrophage colony-stimulating factor. On day 3, 5 ml of this medium was added to the bone marrow-derived DC (BM-DC) culture. On day 8, nonadherent cells were collected and adherent cells were harvested using 10 ml of PBS–5 mM EDTA for 5 min. BM-DCs were counted and incubated at $2 \times 10^6$ cells per ml in complete medium with 1 μg/ml of CyaA-E5, CyaA-E5-Tat, and CyaA-wt. BM-DCs alone in complete medium served as controls. At various times, cells were collected, washed, and prepared for cytofluorimetry. The annexin V-fluos staining kit (Roche Diagnostics, Mannheim, Germany) was used to identify apoptotic cells by following the manufacturer's instructions. Cells were washed and transferred into Falcon 2052 fluorescence-activated cell sorter (FACS) tubes (Becton Dickinson, San Jose, CA), and data from 10,000 BM-DCs cells were collected on a FACScan flow cytometer (Becton Dickinson). Cell death was quantified using CellQuest software.

## RESULTS

**Characterization of the biological properties of the recombinant detoxified CyaA carrying HIV-1 Tat protein.** As a derivative of the CyaA-E5 vector (26), we designed a recombinant CyaA that carries the full HIV-1 Tat protein (CyaA-E5-Tat) inserted at one permissive site located between residues 224 and 225, within the catalytic domain. The protein was expressed in *E. coli* and purified close to homogeneity as indicated by SDS-polyacrylamide gel electrophoresis analysis (data not shown). The presence of the Tat protein was confirmed by Western blot using an anti-Tat monoclonal antibody (data not shown). A lipopolysaccharide elimination procedure (see Materials and Methods) allowed us to obtain CyaAs containing less than 1 EU/μg of endotoxin (data not shown). As expected, the recombinant protein did not exhibit cyclic AMP-synthesizing activity (data not shown) but displayed a weak hemolytic activity (analyzed on sheep erythrocytes as previously described) (61), similar to that of wild CyaA or CyaA-E5. This suggests that the overall structure of CyaA-E5-Tat had not been modified by the insertion of the Tat protein (data not shown). Then we investigated whether the insertion of the Tat protein into CyaA had altered its ability to bind to CD11b-expressing cells using a competitive assay as previously described (20). CyaA-E5 and CyaA-E5-Tat molecules were incubated with transfected CHO cells expressing the human CD11b/CD18 (CHO-$CD11b^+$ cells) and were tested for their capacity to compete for the binding of biotinylated CyaA (CyaA-biotin) to these cells. Both CyaA-E5 and CyaA-E5-Tat inhibited CyaA-biotin binding to CHO-CD11b cells in a dose-dependent manner (Fig. 1A), demonstrating that the insertion of Tat into CyaA did not modify its interaction with CD11b.

To investigate the transactivating capacity of CyaA-E5-Tat, we tested its ability to activate the production of β-Gal in P4 cells. The incubation of the Tat protein with these cells provokes the expression of the *Escherichia coli lacZ* gene, which is under the control of the HIV LTR (48). P4 cells were incubated for 2 h with recombinant Tat or with CyaA-E5-Tat. P4 cells cultured alone served as negative controls. After elimination of the supernatant, cells were cultured overnight with fresh medium and then analyzed for β-Gal production measuring optical density at 595 nm ($OD_{595}$). As shown in Fig. 1B, Tat strongly increased β-Gal production by P4 cells due to its transactivating function. In contrast, CyaA-E5-Tat was unable to induce β-Gal expression (Fig. 1B). This result demonstrates that the Tat protein inserted into CyaA had no transactivating activity.

Then we studied the apoptosis induced by these CyaAs on BM-DCs to analyze the potential toxicity of these molecules. BM-DCs cultured in medium alone served as negative controls. As markers of apoptosis, annexin V and propidium iodide (PI) staining on BM-DCs is shown in Fig. 1C. In the presence of 1 μg/ml of CyaA-E5 or CyaA-E5-Tat, the percentages of annexin $V^+$, annexin $V^+$ $PI^+$, or $PI^+$ BM-DCs were similar to those observed with BM-DCs cultured in medium alone throughout the kinetics (Fig. 1C). Neither apoptosis (as measured by annexin V labeling) nor necrosis (assayed by PI labeling) was observed in BM-DCs incubated with CyaA-E5 and CyaA-E5-Tat compared with BM-DCs incubated in medium alone (Fig. 1C). On the contrary, annexin V staining was expressed by 46% of the BM-DCs as soon as 2 h after incubation with 1 μg/ml of CyaA-wt. By 24 h, around 66% of the CyaA-wt-treated BM-DCs were $PI^+$ cells. Thus, CyaA-wt induces apoptosis of BM-DCs, whereas CyaA-E5 or CyaA-E5-Tat is not toxic for these cells.

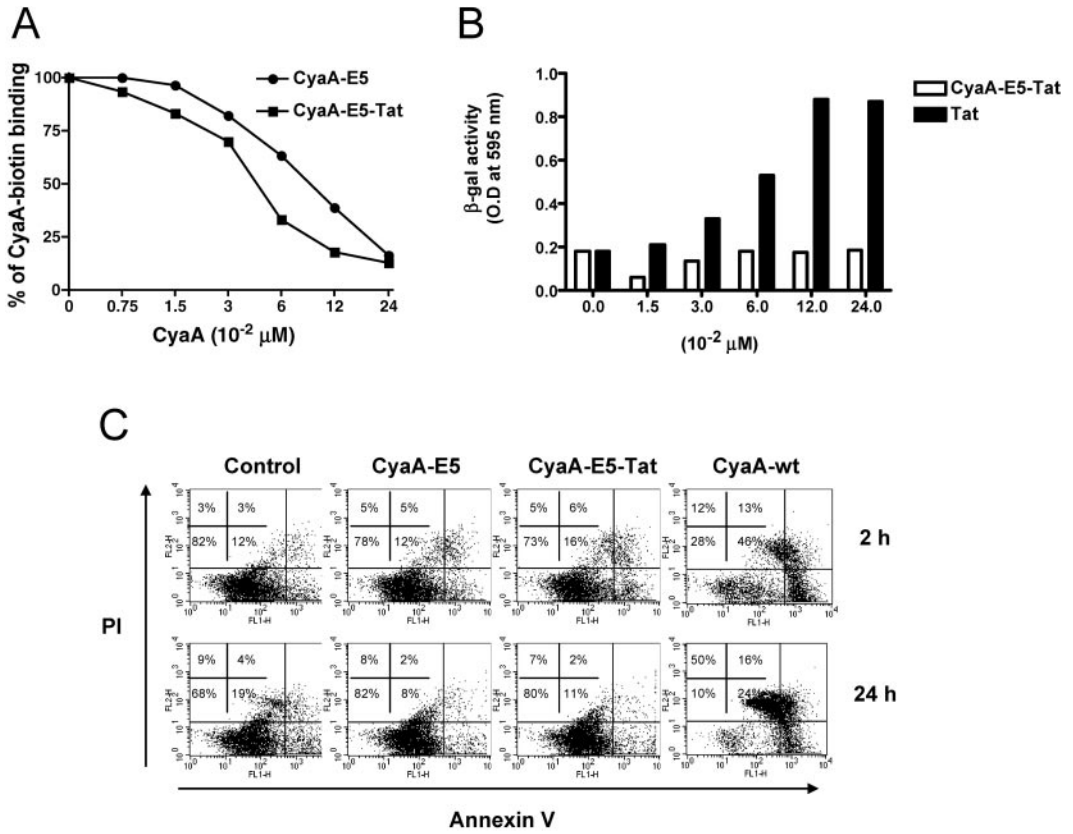


FIG. 1. Analysis of the biological properties of CyaA-E5-Tat. (A) CHO-CD11b cells were incubated for 1 h with indicated concentrations of CyaA-E5 (circles) or CyaA-E5-Tat (squares). Then CyaA-biotin ($3 \times 10^{-2}$ μM) was added in the continuous presence of the recombinant CyaAs. After 30 min, the binding of CyaA-biotin to CHO-CD11b$^+$ cells, measured by FACS, was deduced from the mean fluorescence intensity. Results are expressed as percentages of binding (sample binding/maximum binding) × 100. Data are representative of the results from four independent experiments. (B) P4 cells were incubated with the recombinant native Tat or with CyaA-E5-Tat for 2 h. P4 cells cultured alone served as negative controls. After elimination of the supernatant, cells were cultured overnight with fresh medium and β-Gal production was quantified as described in Materials and Methods. Results are expressed in as optical densities at 595 nm ($OD_{595}$), and data are representative of the results from two independent experiments. (C) BM-DCs from BALB/c mice were generated as described in Materials and Methods, and $2 \times 10^6$ cells/ml were incubated either with medium or with 1 μg/ml of CyaA-E5, CyaA-E5-Tat, or CyaA-wt. After 2 or 24 h, cells were collected, prepared for annexin V/PI staining, and analyzed by FACS to identify apoptotic cells. Results are shown in dot plot format (10,000 events/quadrant). The numbers in each quadrant refer to the percentage of cells. Data are representative of the results from five independent experiments.

**Immunization with HIV-1 Tat delivered by CyaA induces a strong and long-lasting anti-Tat humoral response.** The capacity of recombinant CyaAs to deliver single epitopes to the MHC class I and II pathways to induce CTL and Th responses has been extensively described previously (16, 27, 28, 39, 45, 47, 60). In contrast, whether CyaA could deliver a whole antigen to the immune system to induce humoral response remained to be assessed. To address this issue, BALB/c mice were immunized i.p. at days 0, 20, and 40 with CyaA-E5-Tat (10 μg). Mice immunized with CyaA-E5 (10 μg) served as negative controls. Mice were bled at days 19, 28, and 48, and sera were analyzed for the presence of specific anti-Tat IgG antibodies by ELISA. As shown in Fig. 2A (upper panel), i.p. immunization with CyaA-E5-Tat provoked a rise in the antibody titers after the first injection (6 of 10 mice). Interestingly, after a single boost, all the mice immunized with CyaA-E5-Tat developed an anti-Tat antibody response with a statistical median of antibody titers of log 4 (Fig. 2A, center panel). In addition, a second boost increased the median value up to log 4.5 (Fig. 2A, lower panel).

To evaluate an alternative route of vaccine administration, we studied humoral responses to CyaA-E5-Tat after s.c. immunization. We also compared the immunogenicity of Tat delivered by the CyaA vector to that of an inactive form of Tat (Tat toxoid) that was obtained by alkylation of all cysteine residues of wild-type Tat. On a molecular basis, the dose of 10 μg of CyaA-E5-Tat used in these experiments is equivalent to 0.5 μg of Tat. Thus, the doses of 1 μg or 10 μg of Tat toxoid selected for this comparison were, respectively, 2 and 20 times higher than that utilized to immunize mice with CyaA-E5-Tat (10 μg).

BALB/c mice were immunized by the s.c. route at days 0, 20, and 40 with CyaA-E5 (10 μg), CyaA-E5-Tat (10 μg) alone, or Tat toxoid (1 or 10 μg) in presence or absence of alum. Mice were bled at days 19, 28, and 48, and sera were analyzed for the specific anti-Tat antibody response by ELISA. Following the first immunization, the anti-Tat humoral response was barely detectable in all groups of mice (Fig. 2B, upper panel). After the first boost with CyaA-E5-Tat, 4 of 10 mice developed an anti-Tat IgG response with titers around log 4 (Fig. 2B, center

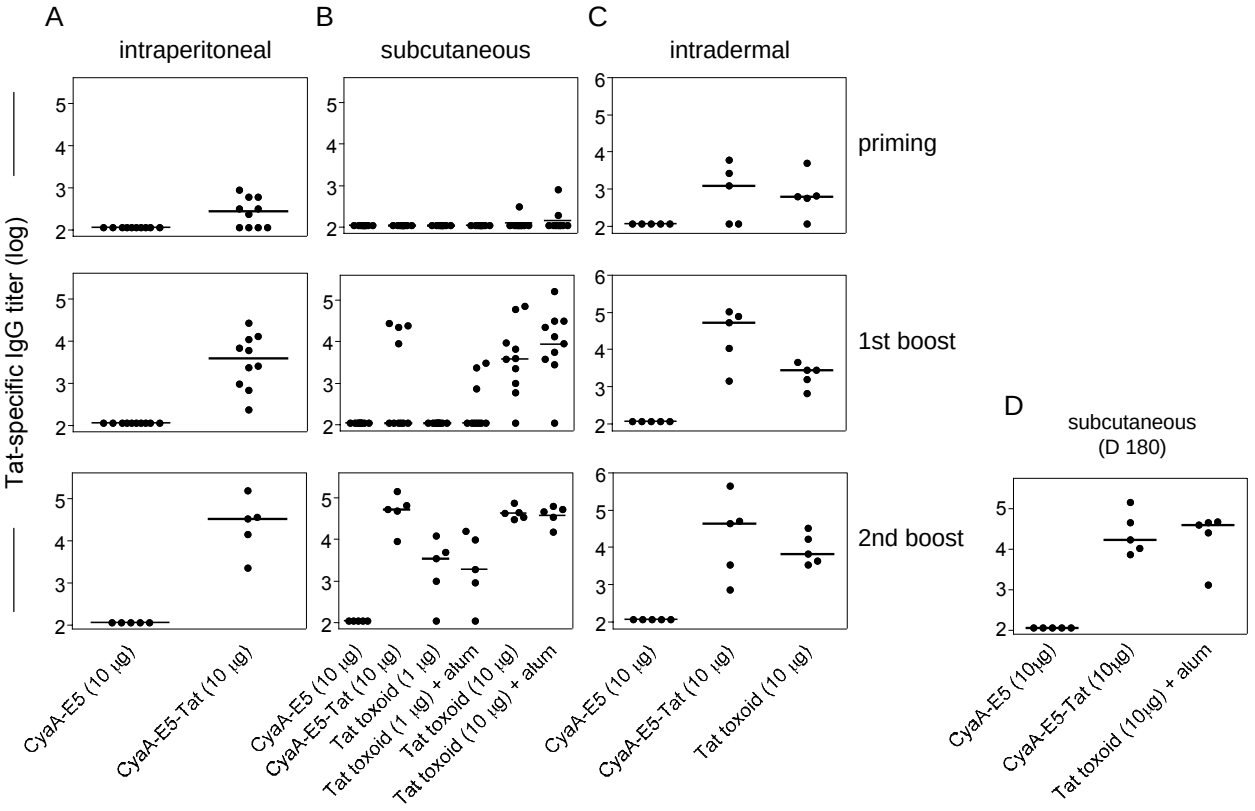


FIG. 2. CyaA-E5-Tat elicits a strong and long-lasting anti-HIV-Tat humoral response. BALB/c mice were immunized at days 0, 20, and 40 i.p. with 10 μg of CyaA-E5 or CyaA-E5-Tat (A); s.c. with CyaA-E5 (10 μg), CyaA-E5-Tat (10 μg), or Tat toxoid (1 μg or 10 μg) in the presence or absence of alum (B); or i.d. with 10 μg of CyaA-E5, CyaA-E5-Tat, or Tat toxoid (C). Upper, center, and lower panels show, respectively, anti-Tat IgG antibody responses of sera from mice bled at days 19 (priming), 28 (first boost), and 48 (second boost) for panels A, B, and C. To analyze the long-lasting anti-Tat humoral responses, BALB/c mice immunized s.c. at days 0, 20, and 40 with 10 μg of CyaA-E5 or CyaA-E5-Tat or 10 μg of Tat toxoid in alum were bled 6 months (day [D] 180) after the last boost and sera were analyzed for specific anti-Tat IgG antibody responses (D). Between 5 and 10 individual sera were analyzed for each group of mice (each point corresponds to 1 mouse). Horizontal bars represent the median responses of each group. Results are representative of four independent experiments.

panel). On the contrary, no humoral response against Tat was detected in mice immunized with 1 μg of Tat toxoid. When alum was added to Tat, low antibody titers were observed (3 of 10 mice) (Fig. 2B, center panel). After the second boost, all mice immunized with CyaA-E5-Tat (5 of 5) (Fig. 2B, lower panel) were responding with a statistical median of antibody titers around log 4.5, whereas a lower antibody response was obtained with mice immunized with 1 μg of Tat toxoid (Fig. 2B, lower panel). A humoral response comparable to that observed with CyaA-E5-Tat alone was obtained when mice were immunized with 10 μg of Tat toxoid with or without alum (Fig. 2B, lower panel). These results demonstrated that Tat delivery by the CyaA vector significantly increased the anti-Tat humoral response compared with the Tat toxoid protein. To reach the same level of response, a 20-fold-higher dose of Tat toxoid protein (10 μg) was needed.

Finally, we analyzed the anti-Tat IgG response after intradermal (i.d.) administration of CyaA-E5-Tat in the dermis of the ear of mice. Indeed, the skin represents a barrier that has an efficient immune surveillance system, which includes Langerhans cells and DCs expressing a high density of CD11b (2). Thus, targeting DCs with CyaA-E5-Tat, by the i.d. route, may produce a more efficient immune response. BALB/c mice were immunized i.d. at days 0, 20, and 40 with CyaA-E5 (10 μg), CyaA-E5-Tat (10 μg), or Tat toxoid (10 μg). Mice were bled at days 19, 28, and 48, and sera were analyzed for the specific anti-Tat antibody response by ELISA. A strong humoral response was induced when CyaA-E5-Tat was injected i.d. even after a single injection (Fig. 2C, upper panel). The antibody titers were further increased after the first and second boosts, reaching a plateau with a statistical median around log 5 (Fig. 2C, center and lower panels). On the contrary, the i.d. injection of a high dose of Tat toxoid (10 μg) failed to induce comparable antibody responses even after two boosts (Fig. 2C, center and lower panels). Thus, immunization with CyaA-E5-Tat by the i.d. route undoubtedly optimized the immune response.

To evaluate the persistence of the humoral response induced by CyaA-E5-Tat compared to Tat toxoid, mice were immunized on days 0, 20, and 40 by the s.c. route with CyaA-E5-Tat (10 μg) alone or with Tat toxoid (10 μg) in presence of alum. Mice immunized with CyaA-E5 served as negative controls. The mice were bled 6 months after the second boost, and these sera were analyzed by ELISA for the presence of specific anti-Tat IgG antibodies. As shown in Fig. 2D, mice immunized with either CyaA-E5-Tat or Tat toxoid maintained a high an-

TABLE 1. Comparaison of the epitopes recognized by anti-Tat antibodies induced by CyaA-E5-Tat and Tat toxoid

| Immunization protocol | Response to Tat peptides containing amino acids[a]: | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1–20 | 21–40 | 36–50 | 46–65 | 56–70 | 65–80 | 73–86 |
| 3× CyaA-E5-Tat (10μg, i.p.) | 3.0 | —[b] | — | 3.8 | — | — | — |
| | 3.0 | — | — | 3.2 | — | — | — |
| | 4.2 | — | — | 4.4 | — | — | — |
| | 4.0 | — | — | 3.9 | — | — | — |
| | 4.0 | — | — | 5.4 | — | — | — |
| 3× CyaA-E5-Tat (10 μg, s.c.) | 5.6 | — | — | 3.8 | — | — | — |
| | 2.6 | — | — | — | — | — | — |
| | 5.5 | — | — | — | — | — | — |
| | 4.2 | — | — | — | — | — | — |
| | 4.2 | — | — | — | — | — | — |
| 3× Tat toxoid (10 μg, s.c.) | 4.6 | — | — | 2.7 | — | — | — |
| | 4.2 | — | — | 2.9 | — | — | — |
| | 4.2 | — | — | — | — | — | — |
| | 4.5 | — | — | 3.4 | — | — | 3.0 |
| | 5.1 | — | — | — | — | — | — |
| 3× Tat toxoid (10 μg) + alum (s.c.) | 4.1 | — | — | — | — | — | — |
| | 5.2 | — | — | 3.1 | — | — | 3.0 |
| | 4.6 | — | — | 4.2 | — | — | — |
| | 4.4 | — | — | 4.2 | — | — | 2.5 |
| | 4.1 | — | — | 3.1 | — | — | — |
| 3× CyaA-E5-Tat (10 μg, i.d.) | 4.9 | — | — | 2.8 | — | — | — |
| | 4.1 | — | — | 4.0 | — | — | — |
| | 3.6 | — | — | 2.6 | — | — | — |
| | 5.4 | — | — | 3.3 | — | — | — |
| | 5.6 | — | — | 3.4 | — | — | — |
| 3× Tat toxoid (10 μg, i.d.) | 4.6 | — | — | — | — | — | — |
| | 3.9 | — | — | — | — | — | — |
| | 4.2 | — | — | — | — | — | — |
| | 4.9 | — | — | 4.1 | — | — | — |
| | 4.1 | — | — | 4.1 | — | — | 2.8 |
| 3× CyaA-E5-Tat (10 μg, s.c.) (day, +180) | 4.7 | — | — | 3.1 | — | — | — |
| | 5.2 | — | — | 3.9 | — | — | — |
| | 4.8 | — | — | 3.6 | — | — | — |
| | 3.8 | — | — | 3.5 | — | — | — |
| | 3.7 | — | — | 3.6 | — | — | — |
| 3× Tat toxoid (10 μg) + alum (s.c.) (day, +180) | 4.3 | — | — | 3.7 | — | — | 2.5 |
| | 3.8 | — | — | 3.8 | — | — | 2.6 |
| | 4.4 | — | — | 5.0 | — | — | 2.5 |
| | 5.3 | — | — | 3.6 | — | — | 3.0 |
| | 2.3 | — | — | — | — | — | 2.5 |

[a] The sera of five mice per group were collected and tested for their response to the indicated Tat peptides. The results are expressed as individual Tat peptide-specific IgG titers (log).
[b] —, no Tat peptide-specific IgG antibodies were detectable.

tibody titer (around log 4.5), demonstrating the persistence of the humoral immune response.

**Mapping of epitopes recognized by the anti-Tat antibodies induced by CyaA-E5-Tat immunization.** Epitope mapping was then performed to determine the B-cell epitope(s) recognized by the anti-Tat antibodies induced by CyaA-E5-Tat immunization compared to those induced by Tat toxoid. This analysis was performed on mouse sera obtained after the second boost following immunization protocols described in the legend to Fig. 2. The Tat peptide containing amino acids 1 to 20 was recognized by all sera from mice immunized either with CyaA-E5-Tat or Tat toxoid (with or without alum), independent of the route of immunization (Table 1). The Tat peptide containing amino acids 46 to 65 was also recognized by some of the mice immunized with CyaA-Tat or Tat toxoid (with or without alum) (Table 1). Particularly, immunization of mice with CyaA-E5-Tat by the i.p. or i.d route led to the induction of antibodies that recognized this peptide. The s.c. immunization also induced antibodies against this epitope but only after long-term immunization, at day 180. When mice were immunized s.c. with Tat toxoid with or without alum, antibodies against the Tat peptide containing amino acids 46 to 65 were detected at day 48 (with alum, 4 of 5 mice; without alum, 3 of 5 mice). The presence of antibodies against this epitope was still observed at day 180 (4 of 5 mice). Finally, the Tat peptide containing amino acids 73 to 86 was only recognized by mice immunized with Tat toxoid (with or without alum) (Table 1). In particular, antibodies against this epitope were detectable, at day 180, in all mice immunized s.c. with Tat toxoid in the presence of alum. However, the reactivity to this peptide was weaker than that observed for Tat peptides containing amino acids 1 to 20 and amino acids 46 to 65.

**CyaA-E5-Tat induces neutralizing anti-HIV Tat immune response.** We then tested the capacity of sera from the vaccinated groups of mice to neutralize the transactivating capacity of Tat on P4 cells. In these cells, the transactivating activity of

TABLE 2. Tat neutralization activity of sera from mice immunized with CyaA-E5, CyaA-E5-Tat, or Tat toxoid

| Serum immunization protocol | % Inhibition of β-Gal-production on day[a] | | | |
|---|---|---|---|---|
| | 48 | | 180 | |
| | 1/20 dilution | 1/40 dilution | 1/20 dilution | 1/40 dilution |
| None (naive) | 16 | 5 | | |
| 3× CyaA-E5 (i.p.) | 27 | 16 | | |
| 3× CyaA-E5 + alum (i.p.) | 18 | 13 | | |
| 3× CyaA-E5-Tat (i.p.) | 74 | 52 | | |
| 3× CyaA-E5 (10 μg, s.c.) | 25 | 19 | 18 | 15 |
| 3× CyaA-E5-Tat (10 μg, s.c.) | 68 | 50 | 63 | 47 |
| 3× Tat toxoid (1 μg) + alum (s.c.) | 39 | 25 | | |
| 3× Tat toxoid (10 μg) + alum (s.c.) | 73 | 57 | 63 | 40 |
| 2× CyaA-E5 (i.d.) | 27 | 13 | | |
| 2× CyaA-E5-Tat (i.d.) | 89 | 64 | | |
| 2× Tat toxoid (10 μg, i.d.) | 59 | 48 | | |
| 3× CyaA-E5 (i.d.) | 32 | 27 | | |
| 3× CyaA-E5-Tat (i.d.) | 83 | 64 | | |
| 3× Tat toxoid (10 μg, i.d.) | 70 | 57 | | |

[a] The sera of five mice per group were collected at the indicated days, pooled, and tested in the neutralization assay. The results are expressed as percentages of inhibition of the β-Gal production that reflects the neutralization of the Tat activity.

the Tat protein can be detected by the expression of the *Escherichia coli lacZ* gene that is under the control of the HIV LTR. P4 cells were incubated with recombinant Tat in the presence of diluted sera from the immunized mice as described in Materials and Methods. The results are expressed as percentages of inhibition of β-Gal production reflecting neutralization of Tat activity. The nonspecific neutralizing activity was determined using sera either from naive mice or from mice immunized with CyaA-E5 in the presence or absence of alum. It represented between 5% and 27% of the Tat activity (Table 2). Sera from mice immunized with CyaA-E5-Tat neutralized between 50% and 89% of Tat activity in a dose-dependent manner. The neutralizing activity of sera from mice immunized with an equivalent amount of the Tat toxoid protein (1 μg) was much lower (Table 2). A noticeable neutralizing activity was also observed following i.d. immunization with CyaA-E5-Tat (89%) and after the first boost (Table 2). On the contrary, sera from mice vaccinated i.d. with 10 μg of Tat toxoid induced a lower Tat neutralizing activity even after two boosts (57 to 70%) (Table 2). The Tat neutralizing capacity of sera from mice that received CyaA-E5-Tat was still detectable 6 months after the last boost (Table 2). Consequently, the capacity of sera obtained from mice immunized with CyaA-E5-Tat to neutralize in vitro the Tat transactivating activity (Table 2) correlates with the strong anti-Tat humoral response (Fig. 2).

**Immunization with CyaA-E5-Tat triggers a long-lasting Th1-polarized response.** To characterize the immune polarization of the responses induced by CyaA-E5-Tat, we analyzed the distribution of the subclasses of the anti-Tat IgG antibodies. The mice immunized with either CyaA-E5-Tat or Tat toxoid by the i.p. or s.c. routes produced IgG1, IgG2a, and IgG2b antibody subclasses, showing no dominant isotype (Fig. 3A and B). When immunized by the i.d. route, the mice receiving CyaA-E5-Tat produced IgG1, IgG2a, and IgG2b antibodies, whereas the mice immunized with Tat toxoid induced only IgG1 antibody responses, suggesting a Th2 polarization (Fig. 3C). We also assessed the distribution of the subclasses of IgG in the sera obtained from mice receiving three doses of 10 μg of CyaA-E5, CyaA-E5-Tat, or Tat toxoid in alum 6 months after the last boost. As shown in Fig. 3D, mice immunized with either CyaA-E5-Tat or Tat toxoid still showed IgG1, IgG2a, and IgG2b antibodies. However, interestingly, the IgG1/IgG2a ratio observed in mice immunized with CyaA-E5-Tat is consistent with a Th1 polarization. In contrast, at 6 months, the IgG1 subclass was the prevalent isotype in mice vaccinated with Tat toxoid, suggesting a Th2-dominant immune response (Fig. 3B, C, and D).

As cytokines play an important role in T-cell polarization, IFN-γ and IL-5 production was then measured in the supernatant from spleen cells activated in vitro. BALB/c mice were immunized at days 0, 20, and 40, and at days 28 and 48, their spleens were removed and a single-cell suspension was prepared. IFN-γ and IL-5 secretion was assessed following in vitro activation with 10 μg of the helper peptide containing amino acids 46 to 65 of the Tat protein (40). As shown in Fig. 4, spleen cells from mice immunized with CyaA-E5-Tat produced IFN-γ (Fig. 4A and C) but no detectable IL-5 (Fig. 4B and D). The specificity of this response was assessed by the lack of IFN-γ production by activated spleen cells from mice immunized with CyaA-E5. Thus, these results indicate that mice immunized with CyaA-E5-Tat developed a Th1-polarized response. In contrast, the splenocytes from mice immunized with either 1 or 10 μg of Tat toxoid in the presence or absence of alum produced both IFN-γ and IL-5. This demonstrates that immunization with Tat toxoid led to a polarization of the immune response toward the Th2 type. Cytokine production was also analyzed 2 and 9 months after immunization with three doses of either CyaA-E5-Tat (10 μg) or Tat toxoid (10 μg) in alum. In mice immunized with CyaA-E5-Tat, spleen cells activated in vitro by the peptide containing amino acids 46 to 65 of the Tat protein produced IFN-γ but no IL-5, indicating a Th1 memory cell polarization (Fig. 5). In contrast, in mice immunized with Tat toxoid in alum, IL-5 was the most prevalent cytokine produced by spleen cells, implying a Th2 memory cell polarization (Fig. 5).

**Immunization with CyaA-E5-Tat elicits HIV-Tat-specific IFN-γ-producing $CD8^+$ T cells, independently of $CD4^+$ T-cell help.** To determine ex vivo the frequencies of Tat-specific IFN-γ-producing T cells, we used a pool of overlapping nonamer peptides of Tat (amino acids 26 to 34, 31 to 39, 36 to 44, and 41 to 49) or a pool of two longer peptides of Tat (amino acids 11 to 24 and 21 to 40) that are known to be targets for CTL in BALB/c mice (49). Mice were immunized intraperitoneally twice with 50 μg of CyaA-E5 or CyaA-E5-Tat in alum as previously described as optimal for CTL generation (26, 28, 59) or with 10 μg of Tat toxoid (26, 28, 59). Seven days later, spleen cells from immunized mice were stimulated in vitro with the pools of peptides (10 μg/ml of each peptide). The number of spleen cells producing IFN-γ was determined by enzyme-linked immunospot assay. Figure 6 shows that both pools of CTL peptide were able to stimulate IFN-γ-producing cells in mice immunized with CyaA-E5-Tat. In contrast, rare IFN-γ-producing cells were detected in mice immunized with Tat toxoid in alum (Fig. 6, median value). As expected, the low

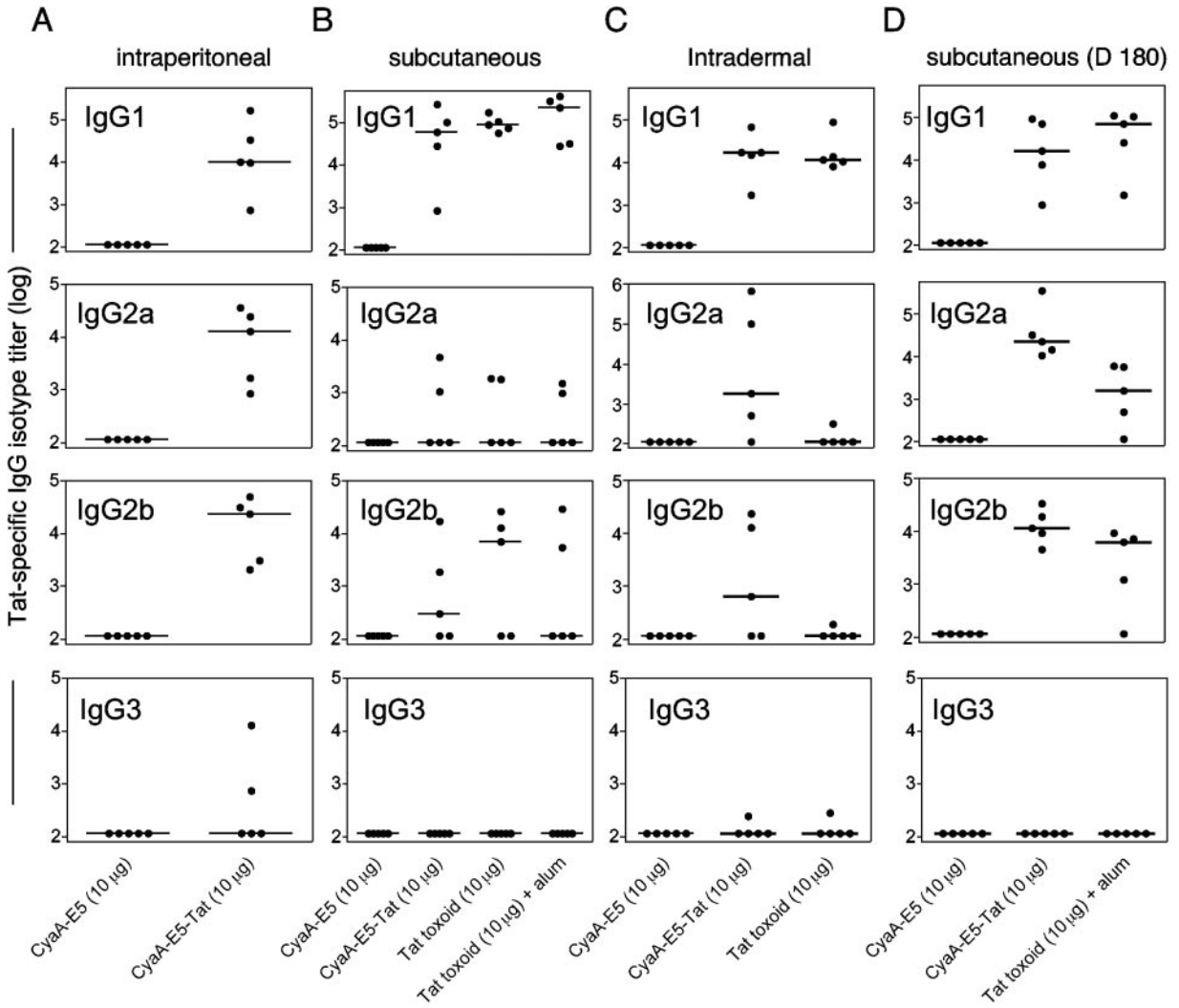


FIG. 3. Analysis of IgG isotypes in mice immunized with CyaA-E5-Tat. BALB/c mice were immunized i.p. (A), s.c. (B and D), or i.d. (C) as mentioned in the legend to Fig. 2. Mice were bled at day 48 (A, B, and C) or 180 (D), and sera were analyzed for the specific anti-Tat IgG isotype (IgG1, IgG2a, IgG2b, or IgG3) antibody response by ELISA. Five individual sera were analyzed for each group of mice (each point corresponds to 1 mouse). Horizontal bars represent the median responses of each group. Results are representative of four independent experiments.

frequency of IFN-γ-producing cells obtained after immunization with CyaA-E5 indicates the specificity of the response.

To evaluate the contribution of $CD4^+$ and $CD8^+$ T cells to the induction of specific IFN-γ-producing cells, we depleted either $CD4^+$ or $CD8^+$ T cells in vivo. Mice were i.p. immunized at days 0 and 20 with CyaA-E5 or CyaA-E5-Tat, and an in vivo depletion of $CD4^+$ or $CD8^+$ T cells was carried out by i.p. injections of rat anti-CD4 or anti-CD8 MAbs on days 28, 29, and 30. Groups of mice receiving control isotype MAbs served as controls. At day 37, spleen cells from immunized mice were stimulated in vitro with the pools of CTL Tat peptides, with the MHC class II-restricted Tat peptide containing amino acids 46 to 65 or with Tat toxoid (10 μg/ml of each peptide or protein). In mice treated with control isotype MAbs, all peptides were capable of stimulating IFN-γ-producing cells after CyaA-E5-Tat immunization (Fig. 7A). In contrast, only the pools of CTL Tat peptides could stimulate IFN-γ-producing cells in the absence of $CD4^+$ T cells (Fig. 7B). This confirmed the capacity of CyaA to induce $CD8^+$ T-cell immune responses as previously described (16, 26–28, 39, 45, 59). As expected, the Tat epitope containing amino acids 46 to 65 and the Tat toxoid still induced IFN-γ-producing cells after depletion of $CD8^+$ T cells (Fig. 7C).

In the perspective of a therapeutic vaccination, we tested whether CyaA-E5-Tat could generate Tat-specific $CD8^+$ T cells in the absence of $CD4^+$ T cells. To address this issue, mice were immunized i.p. at days 0 and 20 with CyaA-E5 or CyaA-E5-Tat concomitantly with an in vivo depletion of $CD4^+$ T cells by i.p. injections of rat anti-CD4 MAbs on days −1, 0,+1, 19, 20, and 21. The mice treated with isotype MAbs served as controls. At day 28, spleen cells from immunized mice were stimulated in vitro either with the pools of CTL Tat peptides or with the MHC class II-restricted Tat peptide containing amino acids 46 to 65 or with Tat toxoid. As expected, Fig. 7D shows that all peptides were able to stimulate IFN-γ-producing cells in mice treated with isotype MAbs and immu-

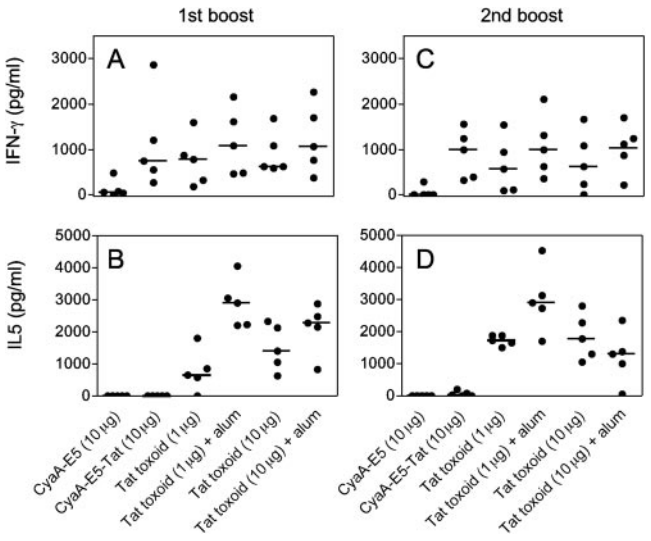


FIG. 4. The HIV-Tat T-cell response induced by CyaA-E5-Tat is Th1 polarized. BALB/c mice were immunized s.c. at days 0, 20, and 40 as described in the legend to Fig. 2B. At day 28 (first boost) and 48 (second boost), spleens were removed and a single-cell suspension was prepared. Cells were activated in vitro with 10 μg of Tat peptide containing amino acids 46 to 65, and 72 h later, IFN-γ and IL-5 production were analyzed in culture supernatants by ELISA. Results are expressed as the concentration of IFN-γ and IL-5 released in the supernatant from duplicate wells. Backgrounds obtained with nonactivated spleen cells were subtracted. Horizontal bars represent the median responses of each group (each point corresponds to one mouse). Left panels: concentration of IFN-γ (A) and IL-5 (B) at day 28 (5 mice per group). Right panels: concentration of IFN-γ (C) and IL-5 (D) at day 48 (5 mice per group). Data are representative of four independent experiments.

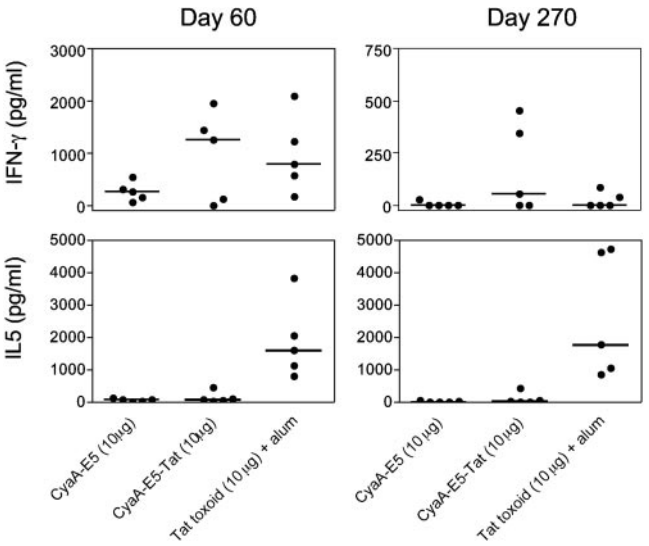


FIG. 5. CyaA-E5-Tat induces long-lasting anti-HIV-Tat cellular responses. BALB/c mice (5 per group) were immunized s.c. at days 0, 20, and 40 with 10 μg of CyaA-E5 or CyaA-E5-Tat or 10 μg of Tat toxoid in alum. At day 60 or 270 after the last boost, spleens were removed and a single-cell suspension was prepared. Cells were activated in vitro with 10 μg of Tat peptide containing amino acids 46 to 65, and 72 h later, IFN-γ and IL-5 production were analyzed in culture supernatants by ELISA. Horizontal bars represent the median responses of each group composed of five individual mice (each point corresponds to 1 mouse). Results are representative of two independent experiments.

nized wiht CyaA-E5-Tat. In CD4-depleted mice, the pool of four nonamers (Tat containing amino acids 26 to 34, 31 to 39, 36 to 44, or 41 to 49) still induced Tat-specific IFN-γ-producing $CD8^+$ T cells (median value or around 10) (Fig. 7E). The number of spots obtained with these mice is comparable to that obtained with mice treated with the isotype MAbs, indicating that $CD4^+$ T cells did not contribute significantly to the induction of the $CD8^+$ T-cell response. Taken together, our data demonstrate that immunization with CyaA-E5-Tat can trigger $CD8^+$ T-cell responses in the absence of $CD4^+$ T cells. These observations may be of critical importance in the context of a therapeutic vaccination of patients with a low frequency of $CD4^+$ T cells.

## DISCUSSION

Several studies have shown that specific anti-HIV-Tat humoral and cellular immune responses in AIDS patients correlated with nonprogression of the viral infection (1, 55, 56, 68, 73). In preclinical studies, the use of vaccines based on native Tat improved both humoral and cellular anti-Tat immune responses (7, 9–11, 49). Nevertheless, due to the possible side effects of Tat (14, 53, 69, 70, 72), administration of active Tat to patients might pose serious safety concerns. Chemically modified Tat-based vaccines have been proposed as an attractive option (18, 52, 67). However, immunization of macaques

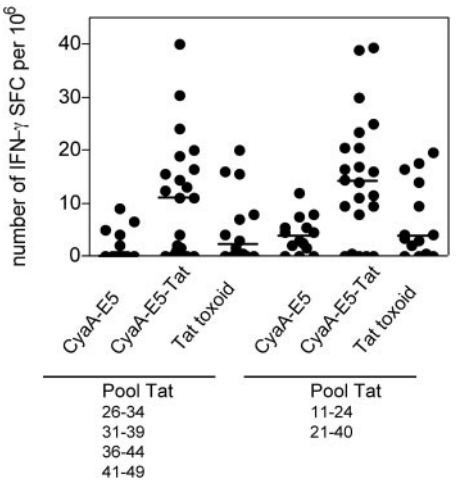


FIG. 6. CyaA-E5-Tat generates HIV-Tat-specific IFN-γ-producing cells. BALB/c mice were immunized i.p. at days 0 and 20 with CyaA-E5 (50 μg), CyaA-E5-Tat (50 μg), or Tat toxoid (10 μg) in alum. Seven days later, spleen cells were stimulated in vitro for 36 h with a pool of overlapping nonamer peptides of HIV-Tat (amino acids 26 to 34, 31 to 39, 36 to 44, or 41 to 49) or with a pool of two longer peptides of HIV-Tat (amino acids 11 to 24 or 21 to 40) in the presence of syngeneic irradiated spleen cells. The results are expressed as numbers of SFC per $10^6$ cells. Horizontal bars represent the median responses of each group (each point corresponds to 1 mouse). Data are the cumulative results from five independent experiments.

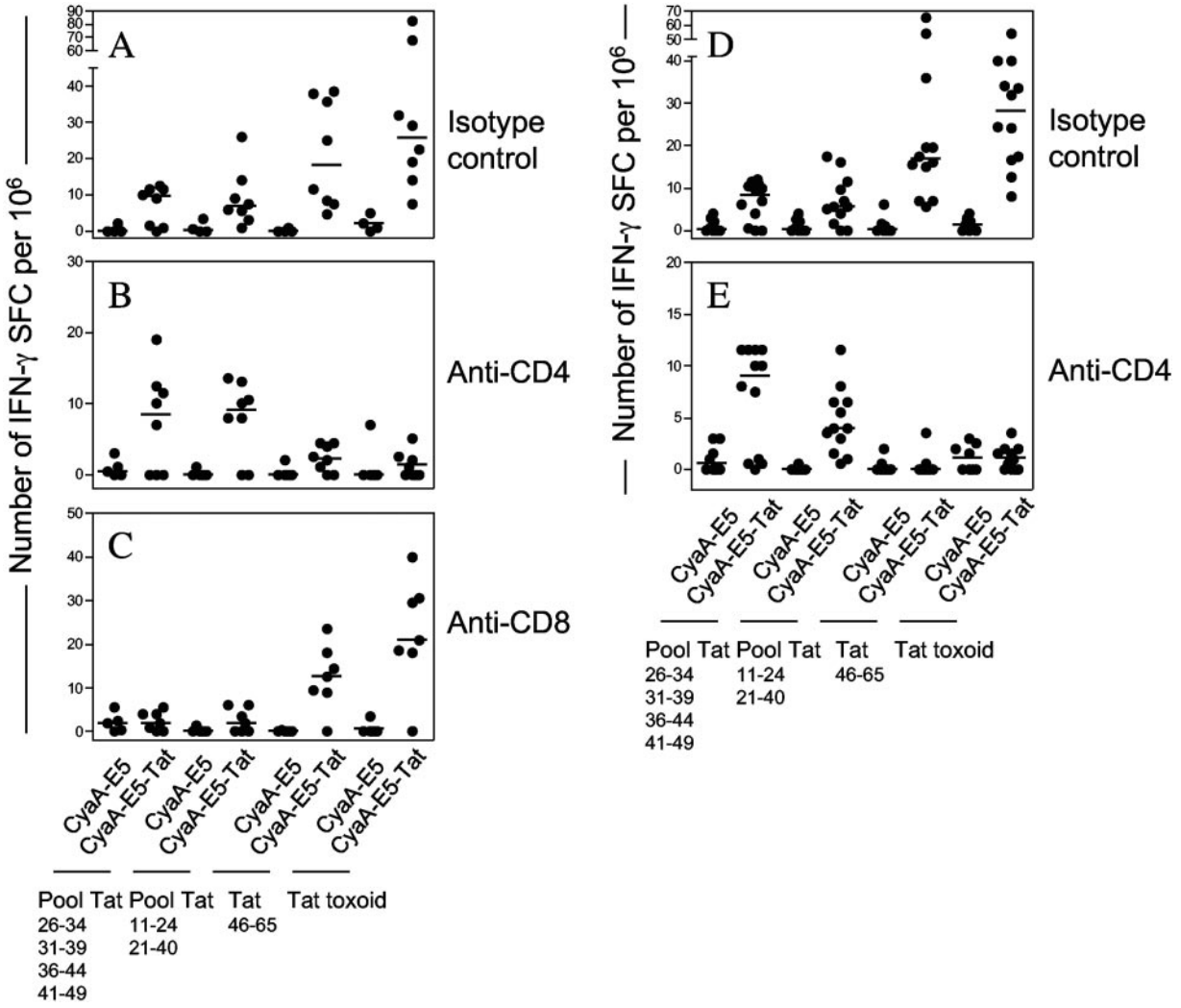


FIG. 7. Immunization by CyaA-E5-Tat induced T-helper-independent HIV-Tat-specific IFN-γ-producing $CD8^+$ T cells. BALB/c mice were immunized i.p. at days 0 and 20 with CyaA-E5 (50 μg) or CyaA-E5-Tat (50 μg) in alum. In vivo depletion of $CD4^+$ or $CD8^+$ T cells was carried out by i.p. injections of rat anti-CD4, anti-CD8, or control isotype MAbs on days 28, 29, and 30 (A, B, and C) or on days −1, 0,+1, 19, 20, and 21 (D and E). Depletion of $CD4^+$ or $CD8^+$ T cells was >95% after treatment with the appropriate MAb (data not shown). At day 37 (A, B, and C) or day 28 (D and E), spleen cells from immunized mice were stimulated in vitro with the pools of CTL HIV-Tat peptides, with the MHC class II-restricted HIV-Tat peptide (containing amino acids 46 to 65), or with Tat toxoid (10 μg/ml of each peptide) in the presence of syngeneic irradiated spleen cells. The results are expressed as numbers of SFC per $10^6$ cells. Horizontal bars represent the median responses of each group (each point corresponds to 1 mouse). Data are the cumulative results from four independent experiments.

with Tat toxoid concomitantly with an adjuvant only attenuated disease (52).

To design a safe and efficient Tat-based vaccine, we have developed a new delivery system using the CyaA vector that targets DCs via the CD11b/CD18 expressed on their membrane (28, 38, 61). Particularly, the CyaA vector can deliver $CD8^+$ and/or $CD4^+$ epitopes to DCs that in turn lead to CTL and/or Th priming via the MHC class I and II presentation pathways (16, 27, 28, 39, 45, 47). In experimental models, the CyaA vector induces protection against viruses and tumors via $CD8^+$ T-cell priming (26, 59). Other new delivery systems that target DCs are currently developed, such as conjugates of antigen to anti-DEC-205 antibodies. Immunization of mice with anti-DEC-205 antibodies coupled to ovalbumin delivered this antigen to DCs and induced strong and specific T-cell responses (6). However, so far, whether antigen delivery to DCs could also induce or potentiate humoral responses has not been analyzed. Our study is the first to demonstrate that this type of strategy, here based on the CyaA vector, can induce both T-cell responses and neutralizing antibodies against antigen delivered to DC.

The CyaA vector carrying Tat shared similar binding ability with CyaA-E5, indicating that the insertion of Tat between residues 224 and 225 of the catalytic domain of CyaA does not alter its interaction with CD11b. This is consistent with our recent data demonstrating that the catalytic domain of CyaA is not involved in the binding to CD11b-expressing cells. More precisely, we located the interacting domain of CyaA in the

glycine/aspartate-rich repeat region (20). The present result suggests that the insertion of Tat in the catalytic domain of the CyaA vector does not affect DC targeting.

Importantly, when inserted into the CyaA vector, the Tat protein displayed no more transactivating activity. This may be due to altered conformation, degradation, or impaired migration into the nucleus of the Tat protein that, consequently, precludes Tat-LTR interaction. We also demonstrated that CyaA-E5-Tat is not toxic to BM-DCs. Thus, the well-established characterization, binding, and biological properties of CyaA toxoid render it an attractive safe vaccine candidate. In this study, we observed that two i.p. or i.d. injections of CyaA-E5-Tat without adjuvant were sufficient to generate strong specific anti-Tat humoral responses that exhibited potent Tat neutralizing capacities. This could be explained by the fact that the CyaA vector targets mostly $CD11b^+$ DCs that are critical APCs for the induction of an immune response. The essential role of DCs in humoral response directly or via T-cell priming has been well documented (5, 19, 30, 42, 43, 54, 65). The efficient humoral response observed after i.d. immunization with CyaA-E5-Tat may be due to the targeting of Langerhans cells and DCs. These cell populations located in the dermis expressed a high density of CD11b and have been shown to migrate to draining lymph nodes after sensitization (2). From a clinical point of view, immunization with CyaA-E5-Tat by the i.d. route may, thus, constitute a pertinent approach.

We also showed that three s.c. immunizations with Tat toxoid in the presence or absence of adjuvant failed to induce a humoral response comparable to that observed with CyaA-E5-Tat when an equivalent amount of Tat was used. A similar response was obtained by the s.c. route only when a 20-times-higher amount of Tat toxoid was utilized. Moreover, i.d. immunization with 10 μg of Tat toxoid can reach neither the level of antibody titers nor the neutralizing capacity obtained in mice vaccinated with CyaA-E5-Tat. It should also be pointed out that CyaA-E5-Tat induces antibodies against B-cell epitopes that are crucial for the activity of the Tat protein. Indeed, the peptides containing amino acids 1 to 20 and 46 to 65 of the Tat sequence are recognized by sera from mice immunized with CyaA-E5-Tat. These two B-cell epitopes, previously identified in BALB/c mice (7, 8, 11), are also recognized by monkey and human sera (46, 64, 66). Interestingly, anti-Tat antibodies against the N-terminal activation domain (amino acids 1 to 20) and the basic region (amino acids 46 to 65) of the Tat protein prevent the HIV-1 Tat transactivating capacity as well as HIV-1 replication and infection (8, 15, 50). Taken together, our results show that immunization with Tat delivered by the CyaA vector without adjuvant induces a high Tat-specific antibody production, demonstrating its capacity to be an efficient vector to generate a long-lasting and neutralizing humoral response. Th1 cellular responses play a key role in protection against pathogens, whereas Th2 polarization increases susceptibility (reviewed in reference 51). Particularly, a switch from the Th1 to the Th2 type in the immune polarization is observed during HIV infection (4, 12, 13). Thus, the induction of Th1-polarized immune responses might be a key aspect to the development of prophylactic or therapeutic vaccines against HIV. Both IgG isotype and cytokine patterns clearly showed that CyaA-E5-Tat induces a Th1-polarized cellular response, as observed with other recombinant CyaAs (17, 60). In contrast, the Tat toxoid administrated with or without alum leads to a polarization of the immune response toward the Th2 type. These results suggest that the polarization is independent of the antigen inserted in the CyaA vector, which is an important observation in view of the development of CyaA as an antigen delivery system.

In addition, we showed that CyaA-E5-Tat can also generate IFN-γ-producing cells using pools of peptides of Tat that were known to be targets for CTL in BALB/c mice in the context of mucosal vaccination (7, 49). In contrast, two vaccinations with Tat toxoid in alum did not result in a statistically significant stimulation of Tat-specific IFN-γ-producing cells. The depletion of $CD4^+$ T cells after CyaA-E5-Tat vaccination confirmed the capacity of CyaA to induce $CD8^+$ T-cell immune responses as previously described (16, 26–28, 39, 45, 59). Finally, we demonstrated that CyaA-E5-Tat vaccination induces Tat-specific $CD8^+$ T cells in the absence of $CD4^+$ T-cell responses. This could have important implications for the development of a therapeutic vaccination strategy in immunocompromised patients.

Recently, we showed that strong and long-lasting melanoma-specific cellular responses could be induced in HHD mice expressing the human HLA*0201 class I molecule, demonstrating that the CyaA vector can deliver epitopes in association with human MHC molecules that is the condition for the use of this vector in humans (16). Furthermore, in contrast to traditional vaccination, our results were mostly obtained without using adjuvant, which reinforces the integration of toxoid CyaA in the design of various vaccines. In the present study, we also proved that CyaA-E5-Tat is capable of eliciting a significant humoral and cellular mediated response superior to the responses obtained with Tat toxoid in the presence of adjuvant. In developing countries, HIV infection is spreading at a high rate, requiring an effective, safe, and low-cost vaccine against AIDS. A vaccine based on Tat delivered by toxoid CyaA that elicits a broad, neutralizing, and long-lasting immune response might thus represent an attractive possibility.

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